STIC-ILL

QR183. J6

From: Huynh, Phuong N.
Sent: Friday, April 27, 2001 3:06 PM
To: STIC-ILL
Subject: RE: 09/684,794

Please deliver the following:

Histochem Cell Biol 2000 Aug;114(2):137-46

J Immunol Methods 1981;46(1):63-8

Hua Xi Yi Ke Da Xue Xue Bao. 1990 Sep;21(3):263-6. Chinese.

J Immunol Methods. 1984 Sep 4;72(2):421-6.

J Immunol Methods. 1993 Aug 26;164(1):141-2. No abstract available.

Thanks,
Neon
Art unit 1644
Mail CM1, 9E12

Journal of Immunological Methods, 72 (1984) 421–426
Elsevier

JIM 03173

Rapid Method of Extraction of Antibodies from Hen Egg Yolk [1,2]

Heike Bade and Hermann Stegemann [3]

Institut für Biochemie, Biologische Bundesanstalt, Messeweg 11, D-3300 Braunschweig, F.R.G.

(Received 9 January 1984, accepted 30 April 1984)

Antibodies were raised in laying hens and isolated from the yolk of their eggs by precipitation with precooled (−20°C) propane-2-ol and removing lipid material with propane-2-ol and acetone. The dried precipitate was extracted with phosphate buffer and shown to contain IgG antibodies and a small amount of additional protein. By Ouchterlony gel diffusion and rocket immunoelectrophoresis, the concentration and quality of specific antibodies in the extracts were comparable to those found in the serum of rabbits or to the IgG separated from yolk by other methods. The new isolation procedure is rapid, reliable and convenient.

Key words: *antibody extraction – yolk IgG – immunized hens – propane-2-ol precipitation*

Introduction

In immunology the production of antisera from the blood of immunized mammals such as rabbits or guinea pigs is most common but the blood of chickens is also frequently used. Like mammals the birds provide their offspring with antibodies (Brambell, 1970) and it has been noted that the immunoglobulin (IgG) content of the yolk is the same or even higher than that in hen's serum (Patterson et al., 1962; Rose et al., 1974). Since it is easy to keep chickens, and since collecting eggs does not require the skilled personnel necessary for the bleeding of animals for serum production, different methods for the isolation of IgG from yolk have been developed (Bar-Joseph and Malkinson, 1980; Polson et al., 1980; Jensenius et al., 1981). The group of Polson et al. (1980) succeeded in isolating a very pure immunoglobulin fraction from yolk but the method, in common with others, is time-consuming. We have developed a rapid method for the isolation of yolk IgG and with no loss of activity in the antibody containing fraction.

[1] This work was supported by the Deutsche Forschungsgemeinschaft (D-5300 Bad Godesberg).
[2] This publication is part of the dissertation of Heike Bade.
[3] Correspondence to: Prof. Dr. H. Stegemann, Institut für Biochemie, Biologische Bundesanstalt, D-3300 Braunschweig, F.R.G.

Materials and Methods

Materials

Brown laying hens, 20 weeks old, from a local farm were kept isolated on regular light cycles. The protein used as antigen ('immature protein') was isolated from immature potato tubers (Stegemann et al., 1973; Stegemann, 1975) by preparative electrophoresis with discontinuous elution in the PANTA-PHOR apparatus (K.H. Müller KG, D-3510 Hann.Münden 1) (Stegemann, 1980). The 'immature protein' was further purified on a Sephadex G-200 column.

Immunization of the hen

The chicken received a first injection into the pectoral muscle with 3.0 mg of the protein, dissolved in 1 ml of 125 mM Tris/boric acid buffer pH 8.9. This solution was emulsified with an equal volume of complete Freund's adjuvant. One week later the hen received a further injection of 3.0 mg of the protein, emulsified in incomplete adjuvant. A booster injection was given after 2 months. The eggs were collected daily, marked for identification and stored for not more than 3 weeks at 1°C until processing.

Extraction of antibodies

Using cooled eggs the yolks were separated from the white and carefully washed with water to remove as much of the albumin as possible. Three to 5 yolks were found to be ideal for one precipitation procedure. The yolk was broken and without the skin slowly poured into precooled (−20°C) propane-2-ol (100 ml per yolk) while thoroughly mixing with a Vibro-mixer (Sartorius GmbH, D-3400 Göttingen). The precipitated protein was allowed to settle for 3–5 min before the supernatant was decanted. For complete removal of the lipids, the sediment was once more thoroughly mixed by vibration for 2 min with the same amount of precooled propane-2-ol as used for precipitation. After sedimentation and decanting of the supernatant this washing procedure was repeated twice with propane-2-ol and twice with 100 ml pre-cooled acetone per yolk. The final residue was collected on a Buchner funnel (filter paper no. 602 h, Schleicher and Schüll, D-3354 Dassel), washed with a small amount of acetone and air-dried. The dry powder was extracted with 0.01 M phosphate buffer pH 7.5 containing 0.1 M NaCl and 0.01% NaN_3 (10 ml per yolk) with stirring for 1 h at room temperature and centrifuged (25,000 × *g* at 10°C for 15 min). The supernatant was stored at 1°C. A further purification was achieved by passing the extract through a DEAE column (5 mm × 23 mm) (Carroll and Stollar, 1983) or by precipitation of the interfering proteins with 12% (w/v) polyethylene-glycol (Fig. 1).

Determination of antibody content

For assessing the titre of the antibodies extracted from the yolks, the Ouchterlony technique was used. In this test various dilutions of the extract ($1:2^n$) in the central wells of an agar gel (1% agar-agar (Serva, D-6900 Heidelberg), with 0.25% NaN_3 and 0.85% NaCl in 0.01 M Tris/HCl buffer, pH 8.0) were diffused against the juice of

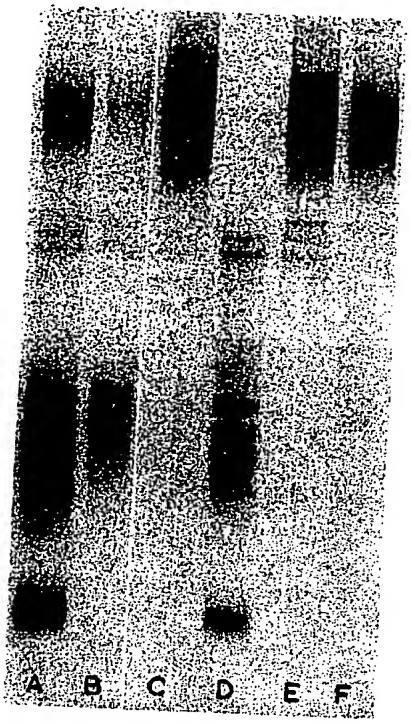

Fig. 1. Electrophoretic evaluation of extracted proteins after different steps of antibody purification. Samples were applied to a 6% polyacrylamide slab gel in 0.125 M Tris/borate buffer, pH 8.9, for 2 h at 500 V and stained with Supranolcyanin 6B (Bayer). A: buffer extract; B: buffer extract purified on DEAE cellulose; C: buffer extract treated with 12% PEG and the precipitated γ-globulins applied; D: supernatant after PEG precipitation; E, F: γ-globulins isolated according to Polson et al. (1980).

immature and of mature potatoes. The highest dilution which still showed a precipitation line was taken as the end-point of the titration. For photographic documentation the immunoprecipitates were stained with Supranolcyanin 6B according to Becker and Sieber (1975). The PANTA-PHOR served for the rocket technique as well.

Results and Discussion

As described previously (Bar-Joseph and Malkinson, 1980; Polson et al., 1980; Jensenius et al., 1981) the raising of antibodies in laying hens and the isolation of antibodies from the egg yolk is a convenient and inexpensive method. We injected the antigen into the pectoral muscle rather than the leg of the chicken in order to minimize discomfort in the bird.

The main problem was the extraction of immunoglobulins from the yolk. Different methods have been developed, but they are either complicated or result in a

product of poor specificity. The aim of our work was to find a more convenient and rapid method for the separation of antibodies from yolk but at the same time achieve a product of satisfactory purity and activity.

The proteins of the yolk were first precipitated by cold propane-2-ol. Then the lipoproteins and the yolk lipids were removed by repeated washes with propane-2-ol and acetone. Other sequences or types of solvents could not be used with the same success. By extracting the light yellow protein precipitate with phosphate buffer pH 7.5 the γ-globulins were dissolved together with some other proteins. The resulting protein solution showed about 10 bands, when analyzed by polyacrylamide gel electrophoresis but the impurities did not disturb the immunological reactions, i.e., rocket electrophoresis. A further purification may be advantageous in special cases. By passing the extract through a very small column of DEAE cellulose (Carroll and Stollar, 1983) or precipitating the γ-globulins with polyethylene glycol type 6000 (Serva) (Polson et al., 1964), most of the impurities can be removed (Fig. 1).

To see whether the extraction was complete, the residue from the first extraction was stirred with fresh buffer for an additional hour. The protein content of both extracts was determined by a modified biuret method (De Wreede and Stegemann, 1981). The first extract contained 10–12% of the total precipitated protein, whereas the second extract contained only 1%. The corresponding ratio of 10 : 1 was obtained by comparing the activities of the first and second extract by the Ouchterlony technique. A second extraction is therefore not needed.

When antibodies extracted by our method were compared with antibodies isolated by the method of Polson et al. (1980) from yolk and with antibodies obtained from sera, no significant differences were noted (Fig. 2). For a comparison of the activities of the antibodies obtained by the new method with the antibodies isolated according to Polson et al. (1980), we first mixed the yolks of 2 eggs and then divided the mixture into 2 equal parts, which were then treated in the appropriate manner. The antibody titres of extracts obtained by the new method were as good or higher than those prepared conventionally (Table I). Furthermore, there was no non-specific reaction when tested against the juice of mature potatoes.

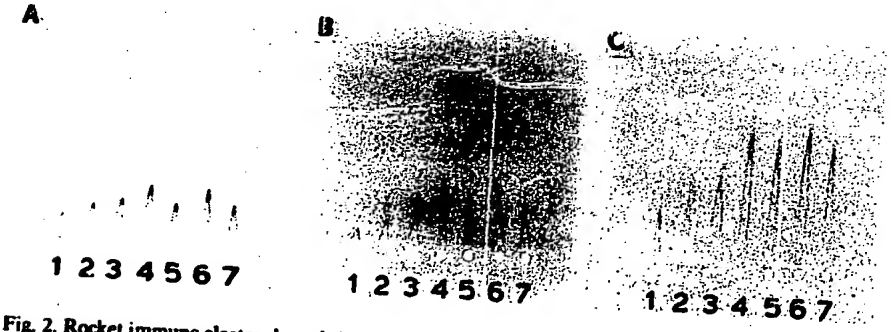

Fig. 2. Rocket immune electrophoresis in 1% agarose in 0.125 M Tris/boric acid, pH 8.9 for 16 h at 40 V. A: rabbit-serum; B: antibody-extract according to Polson et al. (1980); C: antibody extract after propane-2-ol precipitation. Samples: wells 1–3: increasing amounts of 'immature protein'; wells 4–7: juice of immature potatoes.

TABLE I

COMPARISON OF TITRES IN THE ANTIBODY EXTRACTS

Antibody titre determined by Ouchterlony gel diffusion tests.

Antigen	Method	
	Polson et al. (1980)	Propane-2-ol precipitation
'Immature protein'	8 [a]	8
'Immature protein'	8	16
TBSV	512	256

[a] Results expressed as highest dilution giving precipitation with antigen.

Since the 'immature protein' is a weak antigen we checked the method by testing another antibody (raised against TBSV = tomato bushy shunt virus) isolated from the egg yolk of immunized hens. After propane-2-ol precipitation the extracts were compared with material obtained by the method of Polson et al. (1980) not only by an Ouchterlony diffusion test but also by evaluation in an enzyme-linked immunosorbent assay (ELISA). No interfering reactions were seen when tested against buffer control solutions or another virus (PVX). In this test, the sensitivity was somewhat lower for the propane-2-ol extracts (personal communication, Dr. Koenig).

Conclusion

The antibodies isolated from yolk were stable when frozen and after thawing/freezing for at least 3 times, there was no loss in activity. However, the extract contained some other proteins besides γ-globulins and if the removal of these remaining impurities is essential, we suggest passage through DEAE cellulose or precipitation with polyethylene glycol.

Acknowledgments

We are grateful for the valuable advice of Dr. R. Koenig and for the immunization of hens by Mr. Kotulla both from the Institut für Viruskrankheiten in Pflanzen, Biologische Bundesanstalt, Braunschweig.

A grant from the Deutsche Forschungsgemeinschaft is acknowledged.

References

Bar-Joseph, M. and M. Malkinson, 1980, J. Virol. Methods 1, 179.

Becker, W. and A. Sieber, 1975, Methoden der qualitativen und quantitativen Immunelektrophorese (Behring-Institut, Marburg).

Brambell, F.W.R., 1970, The Transmission of Passive Immunity from Mother to Young (Elsevier/North-Holland, Amsterdam).

Carroll, S.B. and B.D. Stollar, 1983, J. Biol. Chem. 258, 24.
De Wreede, I. and H. Stegemann, 1981, Z. Anal. Chem. 308, 431.
Jensenius, J.C., I. Andersen, J. Hau, M. Crone and C. Koch, 1981, J. Immunol. Methods 46, 63.
Patterson, R., J.S. Younger, W.O. Weigle and F.J. Dixon, 1962, J. Immunol. 89, 272.
Polson, A., G.M. Potgieter, J.F. Largier, G.E.F. Mears and F.J. Joubert, 1964, Biochim. Biophys. Acta 82, 463.
Polson, A., M.B. Von Wechmar and M.H.V. Van Regenmortel, 1980, Immunol. Commun. 9, 475.
Rose, M.E., E. Orlans and N. Buttress, 1974, Eur. J. Immunol. 4, 521.
Stegemann, H., 1975, in: The Chemistry and Biochemistry of Plant Proteins, eds. J.B. Harborne and C.F. Van Sumere (Academic Press, London) pp. 71–88.
Stegemann, H., 1980, in: Electrophoresis 1979, ed. B.J. Radola (Walter de Gruyter, Berlin) pp. 571.
Stegemann, H., H. Francksen and V. Macko, 1973, Z. Naturforsch. 28c, 722.